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TREATMENT OF INFLAMMATORY DISEASE

This invention relates to inflammatory disease and more particularly to rheumatoid arthritis.

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the synovial joints which leads to joint destruction, disability and early death. Although the cause of RA is presently unknown, it has been suggested that type II collagen, uniquely found in the articular cartilage, is a possible autoantigen for RA. It has recently been proposed that gp39, a 39KD glycoprotein, and peptides derived from it, are such autoantigens. However, the data supporting this hypothesis are limited and the role of gp39 therefore remains uncertain.

The present invention stems from a different approach based on a study of chondrocytes, the specialised cells of articular cartilage. We have isolated a protein from human chondrocytes and human chondrosarcoma cell lines which reacts with antibodies present in RA patients' sera and meets the accepted criteria for a putative autoantigen. This purified protein has been tested for proliferation of T cells and has been shown to selectively proliferate synovial T cells from patients with RA. This protein is the immunoglobulin heavy chain binding protein BiP(78KD).

International patent application WO 99/18131 proposes the detection of antibodies to a BiP deriving from Hela cells as a diagnostic indication of RA. However, this prior disclosure does not describe the extraction of BiP from the Hela cells in a reproducible manner and is therefore insufficient for practical application.

We have now obtained and identified the correct RA autoantigen and this discovery leads to the development of prognostic and diagnostic tests for this disease and specific therapy. We have isolated and sequenced the DNA for this protein. We have also cloned and expressed this DNA. The amino acid and DNA sequences are novel and are shown in Sequence Listings appended to this specification. The aminoacid sequences of the BiP protein are given as sequence listings in two versions SE1 and SE2 either of which may be used as a test reagent in accordance with the present invention. The cDNA for SE1 is given as Sequence listing SE3. This sequence has been deposited with GENBANK under Accession No AF 188611.

A comparison of this sequence with that of GENBANK Accession No X87949 is provided hereinafter.

The first part of the following description concerns the characterisation of such an autoantigen; the second the cloning, sequencing and expression of the protein; and the third part the demonstration of disease (rheumatoid arthritis) and tissue (synovial compartment) T cell specificity to the autoantigen.

Part 1: CHARACTERISATION OF AUTOANTIGEN

Chondrocytes/chondrosarcoma cells.

Chondrocytes were isolated from cartilage obtained during joint replacement. The cartilage was minced finely and digested with 1mg/ml collagenase (Worthington). Following digestion the cells were centrifuged at 300g and resuspended in Dulbeccos minimal essential medium (DMEM)(Life Technologies, Paisley, UK) enriched with 10% foetal calf serum (FCS)(Harlan Sera-Lab, Loughborough, UK). Cell debris was washed off the adherent cells after 24hours and the cells allowed to expand until confluent. Cells were passaged using trypsin (0.25%) and split 1:3.

Chondrosarcoma cells (HTB94) (SW1353) were supplied by the American Type Culture Collection ATCC (Rockville, Maryland, USA) and by Dr J Block, Rush University, Chicago, USA (personal gift). These cells were cultured in DMEM with 10% FCS and split 1:3 after gentle trypsinisation (0.25% trypsin Life Technologies, Paisley, UK) when confluent.

Preparation of cell lysates.

Cells were scraped from the flask surface, homogenised and sonicated in the presence of proteinase inhibitors PMSF (2mM), leupeptin (200µg/ml) and aprotinin (50µg/ml) (Sigma, Poole, UK).

Sodium dodecyl sulphate (SDS) (Sigma, Poole, UK) was added to a final concentration of 1% and the proteins solubilised at room temperature for

1hour. Protein concentration was estimated by bicinchoninic acid assay using bovine serum albumin (BSA) as a standard protein (Sigma) and the cell lysate was used at 10µg/well equivalent.

Polyacrylamide gel electrophoresis (PAGE) and Western Blot.

The Mini Protean system (BioRad Laboratories. Hemel Hempstead, UK) was used to run the gels. 5, 7.5 or 10% SDS polyacrylamide denaturing gels 1.5mm thick were prepared (See Appendix 1). Gels were loaded with 10µg protein/well or the equivalent was loaded on preparative gels. Electrophoresis was carried out at a constant 100V and broad range kaleidoscope markers (BioRad) were run in parallel with the cell lysates.

Following electrophoresis the proteins were blotted onto nitrocellulose at a constant 100V for 1hour (See Appendix 1). Nitrocellulose was then blocked with 3%BSA (Sigma, Poole,UK) and left at 4^0 C overnight. Preparative gel membranes were cut into 16 thin strips when necessary, each having an identical protein profile. The membranes were probed with patients sera (1/100 dilution) or specific monoclonal antibodies (at required concentrations) for 1hour at room temperature and then washed x3 over 1hour in TTBS (See Appendix 1). The secondary antibody, goat anti-human IgG (Fab^2) horse radish peroxidase(HRP) conjugate (Sigma) was added at 1/1000 dilution and incubated for 1hour at room temperature. The membranes were then washed x3 over 1hour in TTBS. Enhanced chemiluminescence (Amersham) was used to develop the system and antigen-antibody.HRP complexes appeared as discrete bands on photographic film when developed.

Isolation of the putative autoantigen p78

The band of interest was seen in approximately 30% of the rheumatoid arthritis sera used to screen the cell lysates as previously described.

To isolate the protein the cell lysate was concentrated x 23, using a 30,000MW cut-off filter(Vivascience). This protein was then loaded on 5% and 7.5% gels in parallel . One lane on each was loaded at normal concentration while the two other lanes were overloaded with the protein. Kaleidoscope markers were loaded on either side of the test lanes. The gels were then run as previously described until the kaleidoscope markers showed that the 70,000 MW protein would be in the bottom third of the gel as close as possible to the run-off point. The gels were then blotted onto PVDF membrane (Immobilon P, Millipore) which was immediately placed in distilled water after transfer of the proteins was complete. The strip with normal loading was used for immunodetection of the protein band. The developed film of this immunodetection and Ponceau red staining of the overloaded strips was used to identify the band on the membrane which was then air-dried.
These strips were then taken to isolate and sequence the protein using matrix assisted laser desorption ionisation (MALDI) spectroscopy .

The electroblotted proteins were stained with Ponceau S (0.05% w/v aqueous methanol/0.1% acetic acid) using a rapid-staining protocol (1). The dried, stained proteins were then digested in situ with trypsin (Boehringer, modified) and the peptides extracted with 1:1 v/v formic acid:ethanol (2). One 0.2 µl aliquot (approximately 5% of the total digest) was sampled and directly analysed by matrix-assisted laser desorption ionisation (MALDI) time-of-flight

mass spectrometry using a LaserMat 2000 mass spectrometer (Thermo Bioanalysis,UK) (3). A second 0.2µl aliquot was quantitatively esterified using 1% v/v thionyl chloride in methanol and also analysed by MALDI to provide acidic residue composition (4). Native and esterified peptide masses were then screened against the MOWSE peptide mass fingerprint database (5). The remaining digested peptides (90% of total digest) were then reacted with N-succinimidyl-2(3-pyridyl) acetate (SPA) in order to enhance b-ion abundance and facilitate sequence analysis by tandem mass spectrometry (6). Dried peptide fractions were treated with 7 µl 1% w/v N-succinimidyl-2(3-pyridyl) acetate in 0.5M HEPES (pH 7.8 with NaOH) containing 15% v/v acetonitrile for 20 min on ice. The reaction was terminated by 1 µl heptafluorobutyric acid (HFBA) and the solution immediately injected onto a capillary reverse-phase column (300 µm x 15cm) packed with POROS R2/H material (Perseptive Biosystems, MA) equilibrated with 2% v/v acetonitrile/0.05% v/v TFA running at 3 µl/min. The absorbed peptides were washed isocratically with 10% v/v acetonitrile/0.05% v/v TFA for 30 minutes at 3 µl/min to elute the excess reagent and HEPES buffer. The derivatised peptides were then eluted with a single step gradient to 75% v/v acetonitrile/0.1% v/v formic acid and collected in a single 4 µl fraction. The derivatised peptides were then sequenced by low-energy collison-activated dissociation (CAD) using a Finnigan MAT TSQ7000 fitted with a nanoelectrospray source (7,8). CAD was performed using 2.5 mTorr argon with collisonal offset voltages between -18V and -28V. The product-ion spectra were collected with Q3 scanned at 500 amu/sec.

RESULTS:

Sequence data obtained from 7.5% gel (single band)

from GR78_human

Specific identifying peptides:

NQLTSNPENTVFDAK 82-96

SDIDEIVLVGGSTR 353-366

TWNDPSVQQDIK 107-113

Identified human protein GR 78:

Kd glucose related protein precursor (GRP78)

Immunoglobulin heavy chain binding protein (BIP)

Part 2: CLONING, SEQUENCING AND EXPRESSION OF p78

1) mRNA isolation and PCR amplification of identified genes.

Human chondrocytes were isolated and cultured for three weeks as described. Poly(A) mRNA (1-2µg) was extracted with a Micro-Fastrack kit (Invitrogen) from a total of 1-2 X 10^6 cells. One microgram of the resulting mRNA was reverse transcribed into cDNA in a 20µl volume at 45°C for 1 hour using 1µl of Moloney murine leukemia virus reverse transcriptase (200u/µl); 5 X first strand buffer (Tris-HCl pH 8.3, 250mM; KCl 375 mM; MgCl 15mM); 0.1M

DTT; oligo dT(12-18) 20ng/µl (Life Technologies); and dNTP mix 100mM (Amersham Pharmacia Biotec, Uppsala Sweden).

PCR was performed in a 50µl reaction volume under standard conditions (see below) using a Perkin Elmer Applied Biosystems thermal cycler PE2400. Primer sequences were derived from the GenBank database sequence corresponding to the Human gene for the immunoglobulin heavy chain binding protein, Bip (grp78), accession number X87949. Specific primers were synthesized to amplify the putative autoantigen gene from the chondrocyte cDNA. The resulting PCR product consisted of most of the grp78 coding region, bar the untranslated regions, signal sequences and the stop codon (nucleotide positions 279-2169 of the grp78 database sequence).

Primer sets for PCR were designed with integrated restriction sites to allow rapid subcloning of cDNA into the bacterial expression vector. The Forward primers encoded an NdeI site and the Reverse primers contained an XhoI restriction site: The Sequence Listing for the forward primer is given hereafter as SE4 and that for the reverse primer is given as SE5.

Bip Forward primer 5' TATACATATGGAGGAGGACAAGAAGGAGGACG 3' (32mer)

Bip Reverse primer 5' CCACCTCGAGTTCTGCTGTATCCTCTTCACCA 3' (32mer)

After initial denaturation at 96°C for 2min the, PCR was performed for 28 cycles using a cycling profile of 94°C for 30s, 60°C for 30s and 72°C for 2min,

with a final extension at 72°C for 7min. The PCR reaction generated a single specific Bip fragment of 1890bp.

2) Cloning of PCR generated fragments.

The restriction sites engineered into the forward and reverse primers used for the PCR reactions required flanking DNA for them to be recognised by their specific endonucleases (NdeI and XhoI). To provide this flanking DNA, the PCR generated fragment was cloned into a PCR cloning vector pCR2.1-TOPO (Invitrogen). The ligated plasmids were transformed into competent E.coli XL1-Blue (Stratagene) and plasmid DNA extracted using miniprep purification columns (Qiagen). The purified plasmid DNA for the clone was designated Bip-Topo. These DNA samples were stored at -20°C. The purified plasmid DNA for Bip-Topo was digested with NdeI and XhoI. The restricted fragments were separated by agarose gel electrophoresis and purified using the Qiagen DNA gel extraction kit.

3) Subcloning of restricted gene fragments into bacterial expression vector.

The purified fragment for the clone was ligated into the NdeI /XhoI pre-digested bacterial expression vector pET30a (Novagen). Ligation was performed at 12°C overnight in the presence of T4 ligase (20 units) and 1/10 vol of 10X ligase buffer (provided with the T4 ligase enzyme from Promega). The ligated plasmids were transformed into competent E.coli XL1-Blue (Stratagene) and screened by colony-PCR using Bip specific primers. Positive transformants carrying the required recombinant plasmids were purified and transformed into competent E.coli expression strain BL21-(DE3) (Invitrogen).

It will of course be understood that cloning may be carried out in prokaryotic or eukaryotic hosts including bacteria, insect cells, and mammalian cells. Preferred hosts are those which ensure glycosylation of the expressed product.

4) Sequencing of the 1890bp pET30::Bip subclone .

Sequencing of the 5' and 3' terminal ends of the pET30a::Bip clone confirmed that the recombinant DNA molecule was in-frame with the ATG start codon on the pET30 vector and that readthrough from this site continued through the Bip gene and ended with the 6X His residues and the stop codon located on the 3' arm of the expression vector.

Extensive DNA sequencing was performed using synthetic oligonucleotide primers spanning the entire length of the Bip subclone. Sequence analysis of the newly subcloned Bip gene fragment was performed by comparative alignment against the existing grp78 sequence from the database (accession number X87949). A number of differences between the two sequences were detected, both at the DNA and protein level (see Appendix 2). These areas of disagreement may either be a result of errors in the original DNA sequencing (of grp78) or they may indicate the presence of an additional related, but slightly different Bip gene in the genome.

All DNA sequencing was performed on an Applied Biosystems ABI 377 automated DNA sequencer using the dRhodamine dye terminator kit (Perkin Elmer- Applied Biosystems).

Expression of bacterial and purification recombinant proteins.

E.coli expression strain BL21-(DE3) containing the recombinant pET30a-Bip plasmid was grown at 37°C in LB medium containing kanamycin (50μg/ml). When the cells had reached an OD_{600} of 0.6units, isopropyl β-D-thiogalactopyranoside (IPTG) (1mM) was added to the medium to induce expression of the recombinant protein, driven by the IPTG-inducible promoter of the expression vector. To allow for maximal expression of the recombinant protein the culture was incubated for a further 4 hours at 37°C. The cells were pelleted by centrifugation and stored at -70°C.

For purification of the recombinant bacterial proteins the bacterial pellets were lysed in binding buffer (20mM $NaPO_4$, 500mM NaCl, 5mM Imidazole, 1mM PMSF, 1mg/ml Lysozyme, 5units/ml DNAse, 0.1% Triton X-100, pH7.4). The lysate was cleared by centrifugation to remove insoluble matter and cell debris. The cleared lysate was passed over a binding buffer-equilibrated chelating Hi-trap affinity column, with a bed volume of 5ml (Pharmacia). The non-specifically bound protein was washed from the column under stringent conditions using a series of three wash buffers. The primary wash was performed using 100ml of Binding buffer. This was followed by a high stringency low pH wash (20mM $NaPO_4$, 500mM NaCl, 0.1% Triton X-100, pH5.5) and an additional high stringency wash using 100ml of 20mM $NaPO_4$, 500mM NaCl, 0.1% Triton X-100, 50mM Imidazole, pH7.4.

The histidine-tagged recombinant proteins were eluted from the column by stripping with 50mM EDTA. Eluted proteins were dialysed overnight against 1x PBS to remove EDTA and Ni contaminants. The purified protein was

concentrated and washed in sterile PBS using a 50000 Mw cutoff concentrator column (Millipore). The total amount of protein was determined by spectrophotometry using BSA as a standard with the bicinchoninic acid assay.

Immunological studies in experimental arthritis

Antibody response to p78 in experimental arthritis

Collagen arthritis (CIA) and pristane arthritis (PIA) were induced in DBA/1 mice according to our previously described protocol. Mice were bled before induction of arthrtis (15 animals), at the onset of CIA (16 animals) and the onset of PIA (14 animals). The antibody in mouse sera against p78 was determined using an enzyme-linked immunosorbent assay (ELISA) with recombinant p78. Nunc 96-well ELISA plates (Fisher Biotech, Orangeburg, NY) were coated overnight at 4^0C with p78 at 500 ng (in 100 ml of 5% non-fat milk/PBS) per well. After washing 3 times with phosphate buffered saline (PBS) containing 0.05% Tween-20, the plate was blocked with 5% non-fat milk/PBS overnight at 4^0C. The mouse sera were added into the wells at 1:200 dilution in milk/PBS and incubated overnight at 4^0C. The plate was washed, 100 ml goat anti-mouse Ig conjugated with alkaline phosphatase (anti-Ig-AK: 1:500 dilution in milk/PBS/Tween-20, Fisher Biotec) was added for 60 mins at 37^0C. After three washes with PBS/Tween-20, 100 ml paranitrophenyl phospphate solution (PNPP tablets; Sigma Chemicals; St Louis, MO) in diathylanolamine buffer was added to each well. The reaction was allowed to proceed for 30 mins in the dark and the plate read at 405nm in aspectrophotometer (Molecular Devices, Menlo Park, CA). The data were analyzed using the SOFTmax analytical software package. The specific

binding were the OD readings from p78-coated wells subtractinf the OD from non-coated as well as non-sera blanks. The antibody levels were expressed as OD_{405} units.

RESULTS

Identification of autoantigen

When RA and control sera were blotted against chondrocyte extracts, 30% RA sera reacted with a 78 Kd protein compared to 10% of control sera (Figure 1). Sequencing of three tryptic peptides by low energy CAD identified one component of the 78 kD band as the 78 kD glucose-regulated protein, also known as immunoglobulin heavy chain binding protein (BiP). DNA sequence analysis of p78 from articular chondrocyte cDNA showed a number of deviations from the published sequence (accession number X87949). A total of six single nucleotide substitutions and a codon insertion result in three amino acid substitutions and an arginine insertion at position 834-836 of p78 (accession number AF188611).

Immunological tests in rheumatoid arthritis

T cell proliferative responses were determined for mononuclear cell preparations from paired peripheral blood and synovial fluid samples obtained from 23 patients with rheumatoid arthritis and from 12 disease controls. Twelve of 23 (52 per cent) patients with RA and only 2 of 12 (17 per cent) of disease controls showed increased synovial proliferation (Figure 2). The prolifeative response to p78 of RA synovial T cells was significantly higher than that of the paired peripheral blood (stimulation index, mean ± SEM: SF 3.5 ± 0.7; PB 1.6 ± 0.2; $p < 0.01$ Wilcoxon paired test)). A significant difference was also seen between synovial fluid responses to p78 between RA

patients and disease controls (SI: RA 3.5 ± 0.7; OIJD 1.4 ± 0.2; p=0.03 Mann Whitney U test). There was no association with HLA-DR as 50% of responders and non-responders were HLA-DR4 positive (data not shown).

Rheumatoid synovial fluid T cell proliferation to p78 was inhibited by 66-84% by anti-HLA-DR monoclonal antibody L243 (ATCC, Rockville, MD) (data not shown).

No IFNg could be measured in the supernatants from the paired synovial fluid and peripheral blood mononuclear cells (data not shown) despite using an ELISA sensitive to 0.01 ng/ml. No IFNg could be detected by intracellular fluorescence in the peripheral blood T cells of RA patients (n = 7) or healthy controls (n = 2) after stimulation by p78 (data not shown). These findings imply that the responding T cells were unlikely to belong to the classical IFNg producing TH1 subset{1461}.

Immunological studies in experimental arthritis

Induction of experimental arthritis with p78

Immunisation of DBA/1, C57BL mice and Lewis rats with p78 in Freund's complete adjuvant (CFA) did not lead to the development of arthritis (data not shown). There was a similar lack of arthritogenicity of p78 when injected with CFA into HLA-DR1$^{+/+}$ (0/10 mice) or into HLA-DR4$^{+/-}$ (0/5) mice.

Immune response to p78 in experimental arthritis

Despite the failure to induce arthritis by immunising animals with p78, we investigated whether DBA/1 mice during the course of collagen (CIA) or pristane (PIA) induced arthritis developed antibodies to p78 (Figure 4). Mice developed serum anti-p78 antibodies at the onset of collagen arthritis ($O.D_{405}$.

0.189 ± 0.042, m ± sem) and pristane induced arthritis (0.504 ± 0.074) when compared to pre-bleed sera (0.070 ± 0.019; p < versus CIA and p< versus PIA, respectively). Furthermore the concentration of these antibodies was significantly higher in the PIA mice as compared to CIA mice (p). There were 14 mice in each group.

Prevention of collagen-induced arthrtis bv intravenous administration of p78

The presence of antibodies to p78 in the sera of mice with CIA or PIA suggested that manipulating the immune response to p78 might prevent the subsequent development of CIA by a bystander phenomenon. HLA-DR1$^{+/-}$ transgenic mice were injected intravenously with 1mg of p78 prior to immunisation with type II collagen in CFA one week later (Table 2). Whereas 83% of animals had 46% of their limbs involved with arthritis at 8 weeks when pretreated with saline, only 10% of animals had 3% of their limbs involved with arthritis in the group previously given intravenous p78. These differences are highly significant (p≤ 0.008 and p≤ 0.0001). Table 2 also shows that there was a significant reduction in anticollagen antibodies in the p78 pre-treated animals to one third the level in the controls. The reduction was equal in the the IgG1 and IgG2 isotypes (Table 3). The histology of the joints of these animals (Figure 6) confirmed the clinical findings in that there was no synovitis in the joints of p78 pre-treated mice.

We have shown in this study that 30% of patients with RA have antibodies, detected by Western blotting, directed against the human chaperonin BiP/GRP78, and named by us p78. Furthermore, T cells from the rheumatoid synovial fluid proliferated preferentially to p78. There was minimal response by the T cells from the peripheral blood of the same patients. The T cells from

patients with other inflammatory arthritides, whether from the synovial fluid or the peripheral blood, did not significantly proliferate to p78. Finally, the proliferative response was inhibited by anti-HLA-DR monoclonal antibody suggesting that CD4+ positive T cells are responding to antigenic peptides presented in the context of HLA-DR.. This polyclonal T cell response was not HLA-DR4 restricted. Thus p78 stimulation of T cell proliferation possesses two characteristics to be expected of a rheumatoid autoantigen, namely, it is joint and disease specific.

On the basis of these observations, we then undertook intravenous immunisation of mice with p78 in order to test the hypothesis that deviating the immune response to p78 would prevent the development of CIA by a bystander mechanism.. This indeed proved to be the case with almost total prophylaxis against the induction of CIA in HLA-DR1$^{+/+}$ transgenic mice. In the past, various types of experimental arthritis have been prevented or treated by administration of bacterial, and especially mycobacterial, heat shock proteins such as HSP60 or T cells responding to whole HSP60 or to specific peptides{2103, 2280,2282,2283,2284,2281}. However, it is of some importance to note that self-HSP60 peptides show no such protective effects{2281}. Thus the ability of p78 to prevent CIA is of fundamental importance. The observations described in this work are the first, to our knowledge, which implicate an endogenous chaperone in the pathogenesis of RA and the immunotherapy of experimental arthritis. The potential for the immunotherapy of RA is clearly apparent.

2. Use of tests for detection of antibodies to p78 in biological fluids or culture supernatants

Several techniques can be used, such as agglutination, Western blotting, and ELISA.

ELISA protocol for the detection of antibodies to p78 in sera

ELISA plates are coated half with p78 in bicarbonate buffer and half with bicarbonate buffer alone for 4 hours at room temperature. After 2 washes in PBS the plate is blocked for 2 hours at room temperature with 10% goat serum in PBS with 0.05% Tween 20 to stop non-specific binding of protein. After 2 further washes diluted sera (in PBS/1% goat serum/0.05%Tween) is added in duplicate to both the p78 coated and non-coated sides of the plate. After 4 washes biotin conjugated anti human immunoglobulin (1/10000 diluted in PBS/1% goat serum/0.05% Tween)) is added to the plate. The plate is washed 6 times. Bound biotinylated antibody is detected with streptavidin conjugated horse-radish peroxidase (1/800 inPBS/1% goat serum/0.05% Tween) and suitable substrate. Sera containing antibodies to p78 are determined spectrophotometrically. This test forms the basis of a diagnostic and/or prognostic test for rheumatoid arthritis.

3. Therapeutic application

Many routes of administration of the recombinant protein or vector are possible, including intravenous, intramuscular, nasal, oral, cutaneous, and topical. There are several approaches to using p78 or derivatives for therapeutic purposes, including the following:

(a) Induction of mucosal tolerance.

Delivery of p78 autoantigen or peptides derived therefrom by mucosal routes, e.g. through the intestine or nasal mucosa, alters the immune response by downregulating disease activity leaving the patient's immune system otherwise intact. Alternatively p78 or p78 peptides can be delivered as DNA plasmids encoding them with an appropriate mammalian expression vector.

(b) Vaccination with TCR peptides

Peptides of the CDR3 region of the T cell receptor Valpha and Vbeta chains can be synthesised and used as vaccines for delivery by intradermal or intramuscular injection. Plasmids encoding these peptides can be used in the same way.

(c) MHC blockade with native or altered peptides

The p78 peptides may be given parenterally or orally in appropriate cases either unmodified or modified by amino acid substitution and/or attachment of chemical groupings so as to block MHC and especially HLA-DR4 thereby leading to suppression of T cell activation and disease. P78 peptides either native or altered may be combined with soluble HLA-DR4 molecules and applied parenterally or orally.

(d) Induction of tolerance by plasmid DNA immunisation

Plasmids consisting of DNA coding for whole p78 protein or its peptides linked to a mammalian expression vector may be given by injection. DNA coding for human IL-10, IL-4, IL-11, or TGF-beta, incorporated singly or in any combination, may be used to deviate the immune response to p78 towards a TH2 mode so as to suppress disease.

In the therapeutic regimes indicated above the protein or derived peptide may be administered in appropriate compositions delivering amounts ranging from about 0.1 micrograms to about 1 gram or the equivalent in the case of plasmid or vaccine preparations.

Appendix 1

Methodology for gel electrophoresis

Acrylamide gel: 10%

6.075ml acrylamide (40%) (BDH, Poole, UK)
3.35ml methylenbisacrylamide (2%)(Pharmacia Biotech, Uppsala, Sweden)
6.25ml acrylamide gel buffer (see below)
9ml distilled water (produced within the laboratory)
250µl Ammonium persulphate (AMPERS)(0.025mg in 250ul of distilled water)(Sigma-Aldrich, Poole, UK)
25µl NNN'N'-Tetramethylethylenediamine (TEMED)(Sigma-Aldrich, Poole, UK)

Acrylamide gel buffer: pH 8.8
1.5M Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)
pH titration with concentrated hydrochloric acid
0.4% Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

Stacking gel:
1.2ml acrylamide (40%)(BDH-Merck, Poole, UK)
0.65ml bisacrylamide (2%)(Pharmacia Biotech, Uppsala, Sweden)
3.15ml stacking gel buffer (see below)
7.5ml distilled water (produced within the laboratory)

125µl ammonium persulphate (AMPERS) (0.025mg/250µl)(Sigma-Aldrich, Poole, UK)

12.5µl NNN'N'-Tetramethylethylenediamine (TEMED)(Sigma-Aldrich, Poole, UK)

Stacking gel buffer: pH 6.8:

0.5M Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

pH titration with concentrated hydrochloric acid

0.4% Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

Loading buffer:

2ml glycerol

2ml 10% Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

0,.25mg bromophenol blue

2.5ml stacking gel buffer 4-times concentrated (0.5M Tris; 0.4% SDS; pH 6.8)

0.5ml 2-mercaptoethanol (Sigma-Aldrich, Poole, UK)

Electrophoresis/Running buffer:

3g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

14.4g/l glycine (BDH-Merck, Poole, UK)

1g/l Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

Transfer buffer:

3g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

14.4g/l glycine (BDH-Merck, Poole, UK)

1g/l Sodium dodecyl sulphate (SDS)(BDH-Merck, Poole, UK)

14.4g/l glycine (BDH-Merck, Poole, UK)

10% methanol (BDH-Merck, Poole, UK)

Tris Tween buffered saline (TTBS):

2.4g/l Tris (tris(hydoxymethyl)aminomethane)(Sigma-Aldrich, Poole, UK)

29g/l sodium chloride (BDH-Merck, Poole, UK)

500µl tween 20 (polyoxyethylene-sorbitan mono-laurate)(Sigma-Aldrich, Poole, UK)

3% Bovine serum albumin solution (BSA):

3g Albumin bovine fraction 5 (BDH-Merck, Poole, UK)

100ml TTBS (see above)

Appendix 2

Amino acis sequence of the expressed BiP recombinant human protein. Total 1917 nucleotides. Starting at Methionine (start codon) ending in 6X His tag.

Molecular Weight 70937.50 Daltons
639 Amino Acids
86 Strongly Basic(+) Amino Acids (K,R)
108 Strongly Acidic(-) Amino Acids (D,E)
204 Hydrophobic Amino Acids (A,I,L,F,W,V)
142 Polar Amino Acids (N,C,Q,S,T,Y)

5.173 Isolectric Point
-20.041 Charge at PH 7.0

MEEDKKEDVGTVVGIDLGTTYSCVGVFKNGRVEIIANDQGNRITPSYVAFTPEGERLIGDAAKNQLTSNPENTVFDAKRL
IGRTWNDPSVQQDIKFLPFKVVEKKTKPYIQVDIGGGQTKTFAPEEISAMVLTKMKETAEAYLGKKVTHAVVTVPAYFND
AQRQATKDAGTIAGLNVMRIINEPTAAAIAYGLDKREGEKNILVFDLGGGTFDVSLLTIDNGVFEVVATNGDTHLGGEDF
DQRVMEHFIKLYKKKTGKDVRKDNRAVQKLRREVEKAKRALSSQHQARIEIESFYEGEDFSETLTRAKFEELNMDLFRST
MKPVQKVLEDSDLKKSDIDEIVLVGGSTRIPKIQQLVKEFFNGKEPSRGINPDEAVAYGAAVQAGVLSGDQDTGDLVLLD
VCPLTLGIETVGGVMTKLIPRNTVVPTKKSQIFSTASDNQPTVTIKVYEGERPLTKDNHLLGTFDLTGIPPAPRGVPQIE
VTFEIDVNGILRVTAEDKGTGNKNKITITNDQNRLTPEEIERMVNDAEKFAEEDKKLKERIDTRNELESYAYSLKNQIGD
KEKLGGKLSSEDKETMEKAVEEKIEWLESHQDADIEDFKAKKKELEEIVQPIISKLYGSAGPPPTGEEDTAELHHHHHH

Appendix 3

RT-PCR Cloned and Sequenced grp78 (BiP) fragment used for expression of recombinant human protein. Total 1917 nucleotides. Starting at ATG start codon ending in 6X His tag.

ATGGAGGAGGACAAGAAGGAGGACGTGGGCACGGTGGTCGGCATCGACCTGGGGACCACCTACTCCTGCGTCGGCGTGTT
CAAGAACGGCCGCGTGGAGATCATCGCCAACGATCAGGGCAACCGCATCACGCCGTCCTATGTCGCCTTCACTCCTGAAG
GGGAACGTCTGATTGGCGATGCCGCCAAGAACCAGCTCACCTCCAACCCCGAGAACACGGTCTTTGACGCCAAGCGGCTC
ATCGGCCGCACGTGGAATGACCCGTCTGTGCAGCAGGACATCAAGTTCTTGCCGTTCAAGGTGGTTGAAAAGAAAACTAA
ACCATACATTCAAGTTGATATTGGAGGTGGGCAAACAAAGACATTTGCTCCTGAAGAAATTTCTGCCATGGTTCTCACTA
AAATGAAAGAAACCGCTGAGGCTTATTTGGGAAAGAAGGTTACCCATGCAGTTGTTACTGTACCAGCCTATTTTAATGAT
GCCCAACGCCAAGCAACCAAAGACGCTGGAACTATTGCTGGCCTAAATGTTATGAGGATCATCAACGAGCCTACGGCAGC
TGCTATTGCTTATGGCCTGGATAAGAGGGAGGGGGAGAAGAACATCCTGGTGTTTGACCTGGGTGGCGGAACCTTCGATG
TGTCTCTTCTCACCATTGACAATGGTGTCTTCGAAGTTGTGGCCACTAATGGAGATACTCATCTGGGTGGAGAAGACTTT
GACCAGCGTGTCATGGAACACTTCATCAAACTGTACAAAAAGAAGACGGGCAAAGATGTCAGGAAAGACAATAGAGCTGT
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TCTATGAAGGAGAAGACTTTTCTGAGACCCTGACTCGGGCCAAATTTGAAGAGCTCAACATGGATCTGTTCCGGTCTACT
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CAAGAAGTCTCAGATCTTTTCTACAGCTTCTGATAATCAACCAACTGTTACAATCAAGGTCTATGAAGGTGAAAGACCCC
TGACAAAAGACAATCATCTTCTGGGTACATTTGATCTGACTGGAATTCCTCCTGCTCCTCGTGGGGTCCCACAGATTGAA
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AATCACCAATGACCAGAATCGCCTGACACCTGAAGAAATCGAAAGGATGGTTAATGATGCTGAGAAGTTTGCTGAGGAAG
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AAAGAAAAGCTGGGAGGTAAACTTTCCTCTGAAGATAAGGAGACCATGGAAAAAGCTGTAGAAGAAAAGATTGAATGGCT
GGAAAGCCACCAAGATGCTGACATTGAAGACTTCAAAGCTAAGAAGAAGGAACTGGAAGAAATTGTTCAACCAATTATCA
GCAAACTCTATGGAAGTGCAGGCCCTCCCCCAACTGGTGAAGAGGATACAGCAGAACTCCACCACCACCACCACCAC

Appendix 4

RT-PCR Cloned and Sequenced grp78 (BiP) fragment used for expression of recombinant human protein. Total 1917 nucleotides. Starting at ATG start codon ending in 6X His tag.□□ATGGAGGAGGACAAGAAGGAGGACGTGGGCACGGTGGTCGGCATCGACCTGGGGACC
ACCTACTCCTGCGTCGGCGTGTTCAAGAACGGCCGCGTGGAGATCATCGCCAACGATCAGGGCAACCGCATCACGCC
GTCCTATGTCGCCTTCACTCCTGAAGGGGAACGTCTGATTGGCGATGCCGCCAAGAACCAGCTCACCTCCAACCCCG
AGAACACGGTCTTTGACGCCAAGCGGCTCATCGGCCGCACGTGGAATGACCCGTCTGTGCAGCAGGACATCAAGTTC
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TGCTCCTGAAGAAATTTCTGCCATGGTTCTCACTAAAATGAAAGAAACCGCTGAGGCTTATTTGGGAAAGAAGGTTA
CCCATGCAGTTGTTACTGTACCAGCCTATTTTAATGATGCCCAACGCCAAGCAACCAAAGACGCTGGAACTATTGCT
GGCCTAAATGTTATGAGGATCATCAACGAGCCTACGGCAGCTGCTATTGCTTATGGCCTGGATAAGAGGGAGGGGGA
GAAGAACATCCTGGTGTTTGACCTGGGTGGCGGAACCTTCGATGTGTCTCTTCTCACCATTGACAATGGTGTCTTCG
AAGTTGTGGCCACTAATGGAGATACTCATCTGGGTGGAGAAGACTTTGACCAGCGTGTCATGGAACACTTCATCAAA
CTGTACAAAAAGAAGACGGGCAAAGATGTCAGGAAAGACAATAGAGCTGTGCAGAAACTCCGGCGCGAGGTAGAAAA
GGCCAAACGGGCCCTGTCTTCTC
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GTCTGATATTGATGAAATTGTTCTTGTTGGTGGCTCGACTCGAATTCCAAAGATTCAGCAACTGGTTAAAGAGTTCT
TCAATGGCAAGGAACCATCCCGTGGCATAAACCCAGATGAAGCTGTAGCGTATGGTGCTGCTGTCCAGGCTGGTGTG
CTCTCTGGTGATCAAGATACAGGTGACCTGGTACTGCTTGATGTATGTCCCCTTACACTTGGTATTGAAACTGTGGG
AGGTGTCATGACCAAACTGATTCCAAGGAACACAGTGGTGCCTACCAAGAAGTCTCAGATCTTTTCTACAGCTTCTG
ATAATCAACCAACTGTTACAATCAAGGTCTATGAAGGTGAAAGACCCCTGACAAAAGACAATCATCTTCTGGGTACA
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TATTCTTCGAGTGACAGCTGAAGACAAGGGTACAGGGAACAAAAATAAGATCACAATCACCAATGACCAGAATCGCC
TGACACCTGAAGAAATCGAAAGGATGGTTAATGATGCTGAGAAGTTTGCTGAGGAAGACAAAAAGCTCAAGGAGCGC
ATTGATACTAGAAATGAGTTGGAAAGCTATGCCTATTCTCTAAAGAATCAGATTGGAGATAAAGAAAAGCTGGGAGG
TAAACTTTCCTCTGAAGATAAGGAGACCATGGAAAAAGCTGTAGAAGAAAAGATTGAATGGCTGGAAAGCCACCAAG
ATGCTGACATTGAAGACTTCAAAGCTAAGAAGAAGGAACTGGAAGAAATTGTTCAACCAATTATCAGCAAACTCTAT
GGAAGTCAGGCCCTCCCCCAAC
TGGTGAAGAGGATACAGCAGAACTCCACCACCACCACCACCAC□□□□□□□□□□ Original Full length cDNA for Bip (grp78) from database. Accession number X87949. Total length 2554 nucleotides. (previously published)□□aggtcgacgccggccaagacagcacagacagattgacctatt
ggggtgtttcgcgagtgtgagagggaagcgccgcggcctgtatttctagacctgcccttcgcctggttcgtggcgcc
ttgtgaccccgggcccctgccgcctgcaagtcggaaattgcgctgtgctcctgtgctacggcctgtggctggactgc
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ggctgtttactgcttttcattagcagttgctcacatgtctttgggtgggggggagaagaagaattggccatcttaa

Appendix 4 (contd)

aaagcgggtaaaaaacctgggttagggtgtgtgttcaccttcaaaatgttctatttaacaactgggtcatgtgcatc
tggtgtaggaagtttttttctaccataagtgacaccaataaatgtttgttatttacactggtcaaaaaaaaaaaaaaa
aa

Table 1. Prevention of CIA by intravenous injection of recombinant p78

Tolerogen[a]	% Arhritic Mice at 8 weeks[b]	% Arthritic Limbs at 8 weeks[b]	Antibodies (IgG) to CII[c]
p78 (1mg)	1/10 (10%)*	1/40 (3%)**	22 ± 9***
PBS	5/6 (83%)	11/24 (46%).	68 ± 10

[a] HLA-DR1$^{+/+}$ transgenic mice were injected intravenously with either PBS (negative control) or recombinant p78. Either 1mg of protein dissolved in 0.1ml of PBS or 0.1ml of PBS was administered intravenously and mice were immunised with type II collagen in CFA seven days after the intravenous dose.

[b] The incidence of arthritis is reported at 8 weeks after immunisation.

[c] Antibodies represent mean units per group using sera collected 8 weeksafter immunisation.ELISAs were performed and results are reported as units of aactivity derived by cmparison of test sera with the standard serum which was arbitrarily defined as having 50 units of acitivity. Sera were analysed individually and results shown as the mean ± SD for each group of animals.

* p≤ 0.008 (Fischer's Exact test) ** ≤ 0.0001 (Fischer's Exact test) *** <0.05 (Students t test)

Table 2. IgG1 and IgG2 antibody isotypes to type II collagen in mice treated intravenously with either recombinant p78 or PBS.

Tolerogen [a]	IgG1 antibodies to type II collagen [b]	IgG2 antibodies to type II collagen [b]
p78 (1000 μg)	0.71 ± 0.019 *	0.110 ± 0.022 *
PBS	0.135 ± 0.066	0.250 ± 0.044

a HLA-DR1 transgenic mice were injected intravenously with either PBS (negative control) or recombinant p78 followed by immunisation with collagen type II in CFA one week later .

b ELISAs were performed as described in caption to Table 2.

*$p<0.05$ (Students t test)

Table 3: Anti-p78 antibody in CIA or PIA mouse sera (1:200 dil)

Subtract blanks and non-coated values			
Mouse ID	Prebleed	CIA onset	PIA(mouse ID#1-14)
961	0.000	0.243	0.263
982	0.181	0.328	0.981
963	0.155	0.567	0.780
965	0.076	0.198	0.934
966.	0.162	0.257	0.388
967	0.068	0.000	0.291
968	0.039	0.189	0.469
970	0.000	0.000	0.551
197	0.173	0.000	0.537
198	0.023	0.000	0.711
200	0.000	0.099	0.430
248	0.000	0.183	0.535
702	0.090	0.011	0.038
703	0.012	0.206	0.147
705		0.341	
706		0.407	
Mean	0.070	0.189	0.504
Count	14	16	14
SEM	0.019	0.042	0.074

Legends to figures

Figure 1
Western blotting showing 6 rheumatoid sera (lanes 1-6), 5 normal sera (lanes 7-11) and 4 disease controls (lanes 12-15) reacting with chondrosarcoma lysate. Molecular weight markers are shown.

Figure 2
Lymphocyte proliferation in mononuclear cells cultured for 6 days expressed as a stimulation index: proliferation in the presence of p78/proliferation in the presence of culture medium alone. A stimulation of ≥ 2.5 was considered significant. RAPB, rheumatoid arthritis peripheral blood; RASF, rheumatoid arthritis synovial fluid; OIJDPB, other inflammatory joint diseases peripheral blood; OIJDSF, other inflammatory joint diseases synovial fluid.

Figure 3
Antibodies to recombinant human p78 in the sera of mice measured by ELISA and expressed as OD_{405}. Shown are the values for the animals bled before the induction of experimental arthritis (pre-bleed), and at the onset of collagen- induced arthritis (CIA) and of pristane-induced arthritis (PIA).

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